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Protocol for Automated Zooplankton Analysis

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N O T I C E

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16. Abstract (MAXIMUM 200 WORDS)

Ballast water treatment systems must be stringently tested relative to the Coast Guard's proposed ballast water discharge standard for live organisms prior to Coast Guard approval for routine shipboard use. Analysis for live organisms in sparsely populated samples is time-limited and labor intensive. Skilled staff must use a microscope to observe, count and verify viability of live organisms greater than 50 microns in size. An automated method was sought to significantly reduce staff time and effort, maintain consistency, and provide an archive of analytical results. Commercially available laboratory equipment and computer software were used in conjunction with specially developed pattern recognition software. This report provides a description of the equipment required and the protocol developed. A step-by-step protocol is also provided.

The findings in this report are the result of initial research into the potential to automate zooplankton analyses and provide the most current information available at the conclusion of this initial research effort. Additional automation research efforts are ongoing and may affect details reported herein.

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EXECUTIVE SUMMARY

Ballast water is a known pathway for the introduction of non-indigenous species. In an effort to reduce the number of introductions of non-indigenous species into United States waters, the U. S. Coast Guard has proposed regulations requiring ships to meet stringent discharge standards. The proposed concentration for organisms greater than 50 microns is 10 living organisms per cubic meter of ballast water, and this concentration may be reduced if ballast water treatment systems can meet the initial standard. Before the Coast Guard can approve treatment systems for routine use aboard ships, the Coast Guard must verify they are capable of meeting the discharge standard.

A protocol for testing ballast water treatment equipment at full scale has been developed by Coast Guard and the Environmental Protection Agency's Environmental Technology Verification Program. Testing requires evaluating treated samples to determine the number of living organisms present. Current manual methods require skilled personnel using microscopes to observe, enumerate, and determine viability of organisms in concentrated samples within six hours of samples being taken. This visual analysis is labor intensive, requires skilled personnel, is subject to operator fatigue, and provides no archive of results. The Research and Development Center therefore sought a method to automate analyses of the greater than 50 micron (> 50 μm) size class, dominated by zooplankton.

Initial efforts by researchers at the Naval Research Laboratory in Key West, FL had shown that pattern recognition algorithms could be applied to sequential photographs of treated samples to identify motion, and therefore viability, of organisms. Subsequent work refining algorithms, improving equipment, and investigating appropriate stains led to a practical protocol that could be used routinely during tests of ballast water treatment systems.

This report provides background information on how the automation protocol was developed, describes the type of equipment used, and presents the protocol for use by other test facility operators. The text discusses setting up data archives and image collection. The basic protocol is discussed in the text, and a step-by-step protocol for routine laboratory use is provided in an appendix.

This research effort will provide a means to provide consistent analyses of organisms > 50 μm in treated ballast water samples with significantly-reduced, skilled staff-hours. The government developed protocol will be available free of charge in the public domain.

The findings in this report are the result of initial research into the potential to automate zooplankton analyses and provide the most current information available at the conclusion of this initial research effort. Additional automation research efforts are ongoing and may affect details reported herein.

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LIST OF ACRONYMS, ABBREVIATIONS, AND SYMBOLS

BWTE	Ballast water treatment equipment
CMFDA	5-Chloromethylfluorescein diacetate
CCD	Charged coupled device
DIA	Diascopic (brightfield)
FDA	Fluorescein diacetate
GFP	Green fluorescent pigment
GUI	Graphic user interface
m^3	Cubic meter
ml	Milliliter
mm	Millimeter
ms	Millisecond
ND	Neutral density
NRL	Naval Research Laboratory
PC	Personal computer
RGB	Red green blue
X	Magnification power
µm	Micron
<	Less than
>	More than
.nd2	Image file type
.xls	Excel Spreadsheet file type

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1 BACKGROUND AND INTRODUCTION

Testing to evaluate the efficacy of Ballast Water Treatment Equipment (BWTE) requires the characterization of samples to determine the number of live organisms after treatment. Standardized BWTE testing requires that organisms be characterized in three size classes based on maximum dimension on the smallest axis: organisms ≥ 50 microns (μm) (nominally zooplankton), organisms ≥ 10 μm to < 50 μm (nominally protists), and organisms < 10 μm (nominally bacteria). The focus of this document is to provide protocols for collection of image sets, test documentation, image set analysis, and data archive for organisms in the ≥ 50 μm size class. The document also describes the equipment and software required to implement these protocols.

With respect to organisms in the ≥ 50 μm size class, Phase I of the U.S. Coast Guard's proposed discharge standard requires that after treatment, there be less than 10 living organisms per cubic meter (m^{-3}) (Federal Register 2009). It is not feasible to reliably characterize a sparse assemblage of organisms in this large volume of fluid by direct observation. Consequently, statistical arguments require samples to be concentrated (60,000:1) prior to optical evaluation by microscope when determining the efficacy of BWTE. As standardized tests also require that suspended solids and other water properties (e.g., mineral matter and total organic carbon) fall within specified ranges, concentrating samples also increases the amount of suspended and dissolved materials, making these samples significantly more complex to analyze since organisms can be obscured from view by this and other sample debris.

The Naval Research Laboratory (NRL) Center for Corrosion Science in Key West, FL has recently determined that the presence of this and other types of debris (e.g., algal cells) common to BWTE test samples makes it noticeably more difficult to manually count and characterize simple, regular objects (such as 50 μm – 150 μm size polymer beads) in test samples using manual microscopy methods, compared to enumerating these same objects in clean laboratory water. Also, the complexity and diversity of organisms in the ≥ 50 μm size class make it challenging to accurately characterize these samples using manual microscopy methods. Furthermore samples must be analyzed within six hours of being taken.

The motility of organisms in the ≥ 50 μm class presents another challenge, as some of these organisms can move rapidly across a typical sample well or slide in a fraction of a second. Further, suspended solids in the sample can allow these organisms to "hide" or be obscured by debris during observation. The motility of these organisms necessitates the microscope observer view the entire sample well in a single view when conducting analyses. The alternative, magnifying selected regions of the field of view, provides information regarding only organisms in the magnified region (which varies as a result of organism motility during the time of observation).

The requirement to observe the entire sample well can be addressed using a microscope and camera system combination with sufficient spatial resolution. This type of setup can be used to collect an image time series of the sample well. When these systems provide adequate spatial resolution, it becomes feasible to "zoom in" on the individual organisms in the digital image set after the data has been collected from the entire sample well.

NRL has developed and demonstrated the utility of algorithms that use motility to classify the viability of organisms in the ≥ 50 μm size class (Nelson, et al. 2007). This work focused on standard test organisms, including brine shrimp (*Artemia franciscana*) and rotifers (*Brachionus plicatilis* and *B. calyciflorus*). Initial work was conducted with homogeneous monocultures with little to no added debris in the samples.

Towards the end of these feasibility demonstrations, NRL used these algorithms to work with more-complex samples and organism assemblages. This work clearly demonstrated the potential for using motility algorithms to assess viability in complex samples using light microscopy. This work also demonstrated that the microscope and camera systems available at NRL provided sufficient spatial resolution to observe individual organisms (and organism details) in digital image sets in which complete sample wells had been imaged.

NRL has also explored a variety of "vital stains" as a means of determining the viability of protists. Recent work has focused on performing measurements at a variety of geographic locations to demonstrate that these stains provide a location-independent means to identify viable protists in test samples. NRL recommends staining samples with a combination of two vital stains: Fluorescein Diacetate (FDA) and 5-chloromethylfluorescein diacetate (CMFDA, CellTracker™ Green). After entering living cells, non-specific esterases in the cell cleave these stains, resulting in a molecule that fluoresces green when excited with blue light. Work performed at NRL indicates that by using a combination of these two stains, the viability of a greater number of organisms can be determined (compared to using these stains individually). This work also has demonstrated that many organisms' fluorescence signals can be observed even when an organism is obscured by debris. This method, unfortunately, is limited when applied to zooplankton because not all living organisms were stained effectively, and some non-living zooplankters still fluoresced with these vital stains. Nonetheless, using this fluorescence signal in conjunction with motility provides a second means to determine the viability of zooplankton in complex samples.

2 APPROACH

Under this program, NRL developed protocols for the collection, documentation, analysis, and archiving of image data to support BWTE evaluations. This report will first describe the equipment and software required to collect data according to these protocols. This is followed by descriptions of the laboratory procedures used to collect and analyze image data according to these protocols. If other laboratories involved with BWTE testing use the equipment and software specified in this report, then the protocol described in this report should find broad application in supporting standardized testing of BWTE.

It is assumed that samples have been prepared prior to the implementation of this protocol. For zooplankton, this requires that samples first be concentrated to ensure that a sufficient sample volume is analyzed to achieve an accurate analyses of a sparse population of zooplankton (< 10 viable organisms m^{-3}). Ideally samples are concentrated 60,000:1 prior to staining and analysis. Concentrating to a lower level requires that additional samples be analyzed to support accurate analyses.

Following sample concentration, the sub-samples are stained. NRL has been staining its samples using a combination of CMFDA and FDA. The specific procedures that are used for staining samples are described in a 2009 NRL Letter Report (in review as of January 2010). This staining procedure requires a ten minute incubation period prior to sample analysis.

Following staining and incubation, image data can be collected for approximately 20 minutes. Accordingly, the protocol allows images to be collected from a full sample well plate (24 wells) prior to exceeding this 20-minute time-frame.

Based on previous work, all sample concentration, staining and image collection must be completed within 6 hours after obtaining a sample. Analyses can be performed after this time, as they are performed on archived image sets that are collected according to this protocol.

2.1 Equipment

To characterize organisms in the ≥ 50 μm size range with this protocol, it is necessary to use two microscope modalities (i.e., brightfield [white light] and epi-fluorescence measurements) and to apply motility algorithms to both image sets. Combining these measurements allows the user to assess the viability of a wide range of organisms in the complex samples associated with BWTE evaluations. It is also necessary to be able to spatially correlate the image data from both of these modalities. Therefore, image sets from brightfield and epi-fluorescence must be interleaved, not collected in series.

In support of this program, NRL made the modifications required in its microscope/camera systems to simultaneously collect both brightfield and epi-fluorescence time series image sets. This was accomplished using a mechanical shutter that results in the microscope switching between brightfield and epi-fluorescent measurement modes under computer control. In this fashion, this equipment has been used to collect spatially-correlated brightfield and epi-fluorescent images. For the analysis of zooplankton samples, 62 images are collected (31 brightfield and 31 epi-fluorescent) over approximately 32 seconds per sample well.

2.2 Data

Data management and the documentation of system settings are critically important and assure the proper collection of image sets and archiving of test results. The microscope/camera/computer system used at NRL stores all of the image data from a given measurement into a single, large file (denoted as an *.nd2 file). Also, a spreadsheet (with embedded macros and forms) is used by the microscope operator to enter and record information specific to a given test. The operator also fills out specific fields in this spreadsheet to document all of the system settings. The spreadsheet and its embedded forms also provide a checklist to ensure that the operator has made all necessary system adjustments prior to data collection. This test specific Excel spreadsheet maintains this information in an .xls file. Much of these same data are also stored as meta data in the test specific *.nd2 file.

Both of these files have the same name, with two exceptions: the application extension themselves and when sample replicates or sub-samples are collected and analyzed with the same settings. In this latter case, a numeric value is added to the end of *.nd2 file name to indicate the replicate number. Consequently, all data associated with a given test are stored in two files that have very similar names but different application extensions (.xls and .nd2). These files are named in a manner that allows many of the test details (e.g., test date, type of sample analyzed, some system settings, and replicate or sub-sample number) to be discerned from just the file name. This facilitates file recognition and retrieval when reviewing multiple tests.

Data are archived by storing them in a directory with an intuitive structure. First, it separates data by the year, month, and day in which it was collected (each is a separate directory – e.g., C:\2009\9_Sep\24\ ). The Excel spreadsheets described above are stored directly under the day directory. Each *.nd2 file is stored in its own directory. This directory has the same name as the *.nd2 file without the application extension. The file naming and directory structure provides a means to easily access data associated with a given test. Further, the file-naming conventions allow important information about the test to be discerned prior to

analyzing the data. Lastly, the approach used by NRL requires only two files (both automatically generated during the data collection process) to archive all settings, other relevant test information, and the test data (image sets) itself associated with a given microscope measurement. Many of the systems settings are redundantly archived in both of these files, providing additional corroboration of proper system settings.

Data analysis is performed using a stand-alone application developed by NRL. When launched, this application provides a graphical user interface (GUI) that asks the operator to specify the *.nd2 file to be analyzed. The analysis application parses the *.nd2 file, performs all analyses, and stores its results in the same Excel spreadsheet that was used to document test-specific settings. The current focus of these algorithms is to enumerate the number of viable organisms in a test sample. The prototype analysis algorithms developed in previous efforts counted all observed organisms and determined their viability (by detecting movement). The change to enumerating only *living* zooplankters was implemented for two reasons: first, the number of *living* organisms is specified in discharge standards, and second, the increased complexity of BWTE samples rendered it too difficult to enumerate all zooplankters.

3 EQUIPMENT AND SOFTWARE REQUIREMENTS

The imaging system, consisting of the microscope, camera, and control hardware and software (e.g., a personal computer (PC)), is the most critical system to implement the protocol discussed in the next section of the report. The microscope, camera, and accessories described herein are those selected and used at NRL. The overall requirement for system resolution for this equipment is to provide at least 10 µm resolution while imaging a full sample well of approximately 16 millimeters (mm) in diameter. The well should accommodate a sample volume of between 0.5 and 1.5 milliliters (ml). This requirement is to maintain a water column consistent with a microscope's depth of focus when imaging organisms in the $\geq$ 50 µm size class. The microscope system must also be capable of switching automatically between brightfield and epi-fluorescence modalities under computer control. This switching must be accomplished rapidly enough to afford a 1 second interval between successive images that make up the image set.

The Coast Guard does not endorse nor recommend specific equipment or manufacturers. The concepts and algorithms developed in this project were developed for the specific microscope and camera systems resident at NRL. Other equipment with equivalent properties and attributes that provide the resolution and capabilities necessary to support this protocol can be used. However, many key system parameters that are currently monitored by system software may not be recorded as image file metadata with different hardware and operating software. Further, analysis routines will need to be modified to parse image data from a different source and may need additional modification if camera resolution and dynamic range are not identical to those used in the NRL systems.

3.1 Sample Wells

The requirements for the microscope and camera systems are driven largely by the sample well used to analyze samples and its dimensions. The requirement is to collect images of the entire sample well while providing sufficient resolution to allow zooplankton details to be observed in the same images by changing the digital zoom level (the NRL system provides < 10 µm spatial resolution when zoomed in).

NRL performed a series of evaluations on sample well plates. Two major criteria were used: first, it was important that debris in the sample well remain evenly dispersed throughout the well and not move during

the observation. In many of the well plates evaluated, the combination of well shape and materials resulted in debris migrating to the center of the sample well during the observation window (30 seconds). This movement is important, as debris motion can be confused with organism movement by motility algorithms. Second, well plates needed to be constructed of scratch-resistant materials. Based on these criteria, NRL used the SensoPlate™ Glass Bottom Cell Culture Plates (Item # 692892; Greiner Bio-One, Monroe, NC) for analyzing zooplankton samples in the ≥ 50 µm size range. This well tray contains 24 wells, approximately 16 mm in diameter and suitable for containing 0.5 ml to 1.5 ml of fluid. NRL limited the sample volume to 0.5 ml to maintain sample depths consistent with typical microscope depth of focus specifications.

Figure 1 is a photograph of the SensoPlate™ Glass Bottom Cell Culture Plate showing the 24 individual sample wells.

Figure 1. Photograph of the SensoPlate™ Glass Bottom Cell Culture Plate.

3.2 Microscope

NRL worked with Nikon to design a microscope with the desired capabilities for use in conjunction with an existing, NRL-owned, Q-Imaging 1300 Retiga IEEE 1394 camera system. The desired capabilities include:

- Ability to image a 16 mm – 20 mm diameter field of view with < 10 µm spatial resolution.
- Capability to collect images using brightfield illumination and epi-fluorescence.
- Ability to rapidly switch between brightfield and epi-fluorescent modes under computer control.
- Ability to move the sample well tray under computer control so all sample wells on a tray can be imaged.
- Ability to focus the microscope under computer control.
- Ability to monitor key system settings under computer control.

3.2.1 NRL Microscope

NRL used the Nikon Multizoom AZ-100 Multi-Purpose Zoom Microscope. As purchased, this microscope provides basic magnification of 10X, 20X and 50X with an 8:1 variable zoom available at each magnification. Switching magnification is accomplished using a triple nosepiece that allows each of the three objectives to be rotated into position. To support the protocol provided in this report, the microscope is always operated at a basic magnification of 10X with the variable zoom set to 1. Consequently, a single objective could be used to support this protocol. This microscope was originally purchased with the Nikon AZ-FL Epi-Fluorescence Attachment, which allows the microscope to operate in both brightfield and epi-fluorescence modes. The approximate cost of the microscope with the epi-fluorescence attachment (and all required peripherals) was $30,000.

3.2.2 Light Sources

In July 2009, Nikon integrated new light sources that allowed the switching between brightfield and epi-fluorescence under computer control into the NRL microscope. The cost of these light sources and the appropriate control software was approximately $7,500.

3.2.3 Sample Stage

In October 2009, Nikon integrated a computer controllable, motorized X-Y-Z stage to the NRL microscope. This stage allows each sample well on the sample well plates to be moved into position (and image sets collected) under computer control. The stage also provides the capability to focus the microscope under computer control. The cost of this motorized stage was approximately $15,000.

3.3 Imaging and Image Acquisition Software

Nikon NIS-Elements Advanced Research Imaging System Software (Elements) is used to control the microscope system during automated data collection. This software controls the entire image acquisition process, the storing of image data into a single *.nd2 file, the switching of the light sources during image acquisition, and the movement of the motorized stage when multiple sample wells are evaluated in sequence on a single multi-well plate. The software also controls the camera settings and provides image display and image processing capabilities. The cost of this software package was approximately $ 3,600.

3.4 Camera

As mentioned above, NRL used a Q-Imaging 1300 Retiga IEEE 1394 camera. It provides a maximum resolution of 1300 x 1030 pixels using a large area charged coupled device (CCD) detector that provides high light sensitivity (compared with consumer CCD-based imaging systems, such as video cameras). The camera is fully controllable using the NIS-Elements software. Because the camera's detector is no longer manufactured, this camera is no longer available from Q-Imaging and has been replaced by the Q-Imaging 2000 R Retiga IEEE 1394 camera, which provides 1600 x 1200 pixel resolution. As such, it will result in a higher resolution imaging system than the system that is available at NRL. For this application, the monochrome, un-cooled version of the camera is recommended. The cost of this camera is approximately $7,000. An even higher resolution camera (that will result in still more improved spatial resolution) that uses a 2048 x 2048 pixel detector is available at a cost of approximately $ 9,000. Both cameras are fully compatible with the Nikon Elements Software.

3.5 Computer

To complete the system, a PC with at least two RS-232 ports, four USB-2 ports, and an IEEE 1394 (Firewire) interface is required. The cost of an appropriate PC and display for the imaging system is estimated at $ 3,000.

3.6 Settings

Following the October 2009 updates to the microscope system, the majority of system settings are either directly monitored by the computer or entered by the microscope operator into the spreadsheet (as discussed in the next section). Many of the parameters entered into the spreadsheet can be corroborated, as these same data are recorded in the *.nd2 files. There are currently four parameters that are adjusted manually by the microscope operator that can not be corroborated in the *.nd2 files. First, the operator manually adjusts (or ensures) that the variable zoom level is set to 1 for implementation of the data collection protocol. The operator is asked by the spreadsheet to confirm this zoom level prior to the start of data acquisition. Second, the filter cube position is manually set to position 2 – for the Green Fluorescent Protein (GFP) filter cube. Again, the operator is asked by the spreadsheet to confirm the filter cube position prior to the start of data acquisition. Third, the microscope operator needs to ensure that the 1X objective (10X system magnification with the ocular lenses) is used. The operator is also asked to corroborate this point prior to data acquisition. Lastly, the microscope operator needs to ensure that the neutral density (ND) filters are in the proper configuration: the ND2 filter should be not engaged and the ND8 and ND16 filters should be engaged. The microscope operator is asked to corroborate this configuration prior to data collection.

The total cost of a new system for implementing the protocols described in the next section of this report is approximately $66,000 (Table 1). With the upgrades made in its microscope system, the imaging system at NRL is now also configured to implement the protocols provided in the next section of this report.

Table 1. Equipment summary and costs.

Item	Model Used	Approximate Cost
Microscope	Nikon Multizoom AZ-100 Multi-Purpose Zoom	$30,000
Light Source	Nikon Computer-Controlled Light Switcher	$7,500
Sample Stage	Nikon Motorized XYZ Stage	$15,000
Imaging Software	Nikon NIS-Elements Advanced Research Imaging System Software	$3,600
Camera	Q-Imaging 2000 Retiga IEEE 1394 camera	$7,000
Computer	PC with 2 RS-232, 4 USB-2 Ports & IEEE 1394 (Firewire)	$3000
Analytical Software	Government developed	$0
TOTAL		**~$66,000**

3.7 Data Analysis Software

A series of algorithms was developed by NRL to analyze the images collected and stored by the Elements software. The algorithms analyze the images in the selected file and store the results in the file's associated spreadsheet file. This government-developed software for analyzing the collected data will be made available at no cost.

4 DATA MANAGEMENT AND ZOOPLANKTON DATA COLLECTION PROTOCOLS

Two protocols have been developed by NRL to satisfy the requirements of standardized data collection: the Data Management Protocol and the Zooplankton Data Collection Protocol. They are meant to be used in concert and ensure that system settings are properly set, documented, and recorded. As described below, in many cases, redundant information is recorded in the two major outputs: an Excel Spreadsheet, which contains comments by the operator and lists the settings used, and an *.nd2 file, which contains the diascopic (DIA, transmitted light (brightfield)) and epi-fluorescent image sets. NRL has successfully used both protocols to support its data collections since August 2009.

Implementing the protocols is a three step process. First, the operator sets up the directories and generates a test specific Excel spreadsheet. This process is described in Section 4.1 and 4.3 and detailed in Appendix A. The operator next sets up the microscope and collects the image data. This is described in Section 4.2 and 4.3 and detailed in Appendix A. The operator then runs the analysis routines on the collected image set data. This process is described in Section 5.

4.1 Data Management Protocol

The Data Management Protocol serves two purposes. First, it provides a standardized means of storing data with a file naming convention into a directory structure that allows specific test data to be easily identified. Second, it produces a test-specific spreadsheet that documents key system settings and allows the microscope operator to enter test-specific comments following a review of the image set after it is collected.

4.2 Zooplankton Data Collection Protocol

The purpose of the Zooplankton Data Collection Protocol is to ensure that standardized image data sets are collected. It is used to set the microscope and camera settings prior to data collection and to collect the standardized image sets in an *.nd2 file format following system setup. At this time, only two camera setting parameters are selected by the microscope operator: the GFP (green fluorescent pigment) camera exposure time and the gain settings. Based on recent work, it is likely that the protocol will be modified to fix these two parameters at values of 50 milliseconds (ms) and 5 X, respectively. Many other parameters, such as lamp brightness, are automatically set by configuration files (not editable) accessed by Nikon Elements and are not user-selectable. These meta-data are additionally stored in the *.nd2 file.

4.3 Using the Protocols – Summary Steps

Brief descriptions for using the Data Management Protocol and Zooplankton Data Collection Protocol follow. The protocols assume that the microscope operator has basic familiarity with the AZ-100 microscope and the Nikon Elements Software. The complete protocols are provided in Appendix A. They are provided there in the same format that they have been provided to the laboratory staff responsible for data collection at NRL.

Prior to running the management and collection protocols, the sample should have been concentrated and stained. A 0.5-ml subsample should have been placed in the sample well and the sample plate placed onto the sample stage. Note that the stains have a finite lifespan of about 20 minutes.

4.3.1 Data Management – File naming

The observer starts by creating a new experiment folder for each new *.nd2 file. This folder will contain all images taken for each sample well analyzed during the experiment. A strict file/folder naming convention allows all replicate or subsample data to be associated with the corresponding .xls spreadsheet. The folder should be labeled with the date and a sequential number. For example, a folder Desktop > ANS > 2009 > Month > Day > Treatment > Run Folder could be Desktop > ANS > 2009 > 07_July > 09 > rotifer_300ms > 20090709_rotifer_300ms_001. The file itself should be named to match the run folder.

Once the folder name is established, an Excel data sheet template (.xls) is saved to the folder with the .xls file having the same name as the folder. [If necessary, the operator may need to change the Excel security setting to medium to allow Excel to open imbedded macros.] The operator then opens the Excel worksheet and enters appropriate data for the run. All entries, including redundant data, must be filled before saving. Comments can be added to the worksheet after the .nd2 images are reviewed.

4.3.2 Zooplankton Data Collection

With the camera and both lights on, the Elements software is turned on. After the microscope is focused and the optical pathway sent to the camera, the operator opens a live preview in Elements. Clicking the DIA optical configuration on the toolbar allows the operator to focus the camera based on the Live Preview window. The operator then checks and records the physical microscope settings. The operator then changes to the GFP optical configuration on the toolbar and verifies the GFP settings.

Focusing (eye or camera) is not trivial as a result of the depth of the water column, but skilled and experienced microscope operators should be able to accomplish this function. It is important to note that we are recommending a paradigm shift in the way the microscope is operated to focus on the complete well and not on individual organisms. Focus becomes more important here, as magnification and the ultimate resolution of the individual organisms is obtained by zooming in on the images rather than on the organisms.

With all settings verified and recorded, the operator selects which folder to save the images in and enters the first filename as "filename_001". [The Elements software will automatically increment the number at the end of each run.] The operator sets the interval to 1 second and the Duration to 30 seconds. Finally the operator checks the Lambda tab to verify that the first Lambda is GFP and the second is DIA.

The operator then clicks "Run now" to start automatically collecting images of the sample under the ocular. As indicated earlier, this only results in the collection of the image sets. Analysis of the image sets occurs later.

5 DATA ANALYSIS AND BASIC ALGORITHM DESCRIPTION

The Data Analysis algorithms are executed using a zooplankton analysis program as a stand-alone, Windows-based application. (This analysis software will be available at no cost.) When run, the application asks the operator to specify a *.nd2 file to be analyzed. The *.nd2 file associated with a given test is parsed and the motility algorithms operate on the parsed image sets. The algorithm provides a display of the DIA and GFP image set movies and provides a screen output of the number of living organisms detected in the analyzed data. Additionally, the data analysis routine outputs its final (as well as intermediate) counting results directly into the spreadsheet described in the Data Management Protocol.

Initially, the basic analysis algorithm analyzes the brightfield (DIA) and epi-fluorescent (GFP) data independently. The algorithm works with successive image pairs in each microscope modality (e.g., 30 successive image pairs in each DIA and GFP image set (31 DIA and 31 GFP images total)). Thresholds are applied to these images to create binary images that are subtracted and squared to create new binary images, which depict particles that have moved between image pairs. The number of moving organisms is computed on an image-by-image basis for each of the DIA and GFP sets. Next, these results are weighted by performing analysis across successive image pairs (all 30) in each microscope modality and then by analyzing the results across the associated DIA and GFP image pairs (on a spatial bases). The results are then aggregated across the complete image sets to generate the number of living organisms associated with a given analyzed *.nd2 file. As mentioned above, both intermediate and final results are written into the Excel spreadsheet associated with the *.nd2 file analyzed.

Results are written to the Excel spreadsheet associated with the given *.nd2 file that was analyzed. The overall result (the number of viable organism/ml) based on the analysis of the entire image set is written to the spreadsheet. The software additionally writes out interim results that are generated on an image pair by image pair basis for both the DIA and GFP image sets. Lastly, image data are available for review by the operator. This allows the operator to corroborate automatically generated results and, if required, to amend results based upon their observations of the image set data.

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Federal Register (2009) Standards for Living Organisms in Ships' Ballast Water Discharged in U.S. Waters; Draft Programmatic Environmental Impact Statement, Proposed Rule and Notice, 74 FR 44631-44672 (28 August 2009). National Archives and Records Administration, Washington, DC

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APPENDIX A. PROTOCOLS FOR AUTOMATED ZOOPLANKTON ANALYSIS

The protocols on the following pages provide step-by-step instructions for creating data files, capturing images of treated samples, and analyzing the collected images. The protocols and accompanying screen images were developed using the equipment described in the preceding text. Steps include file naming conventions, creation of an Excel data sheet, data collection itself, and analysis of digital zooplankton images. The government-developed software for analyzing the collected data is in the public domain and is available from NRL.

A.1 Zooplankton Data Management Protocol File Naming and Convention

Create a new experiment folder for every new .nd2 file.

Desktop > ANS > 2009 > Month > Day > Treatment > Run Folder

The Experiment Folder should be labeled with the date and then a chronological number. Examples:

Desktop > ANS > 2009 > 07_July > 09 > rotifer_300ms > 20090709_rotifer_300ms_001 (e.g., replicate or subsample 001)

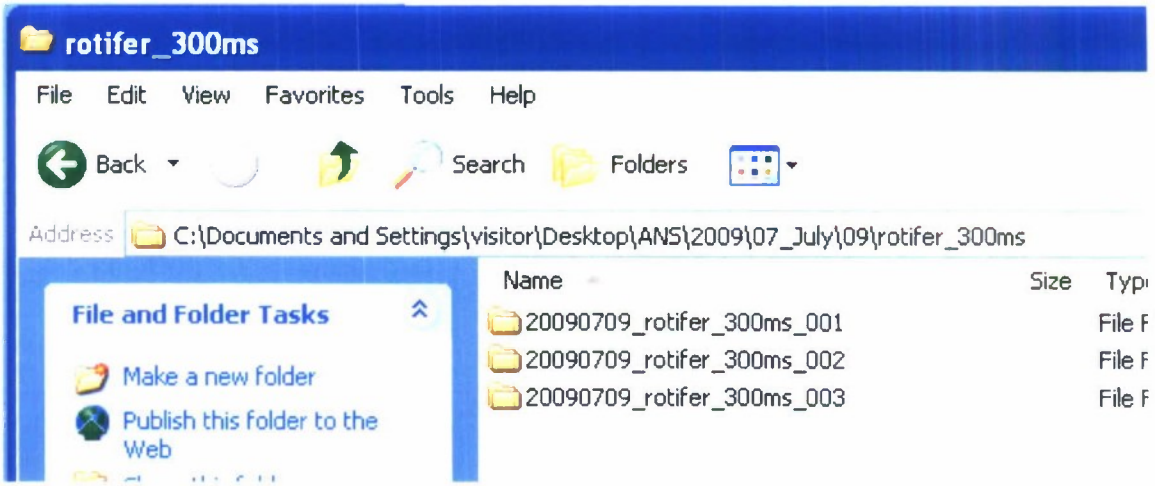

Figure A-1. File naming and convention.

The file itself should be named to match the Run Folder: 20090709_rotifer_300ms_001.nd2 (Figure A-1).

A.2 Zooplankton Data Management Protocol Excel Data Sheet

Each Treatment Folder should have a copy of the AZ100 Excel data sheet.

1. The template will be located in the ANS folder. Save your edited version in the Treatment folder with a filename identical to the Runs (e.g., 20090709_rotifer_300ms.xls). See Figure A-2.

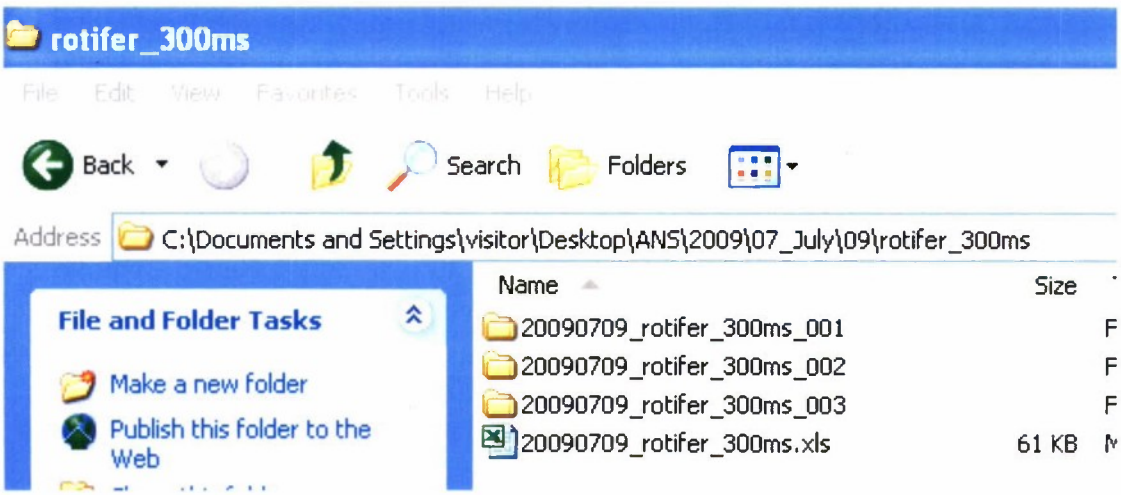

Figure A-2. Excel data sheet in Treatment folder.

2. You may have to configure Excel to be able to open the macro called out in the programs. If a pop-up window asks you if you want to enable macros, say Yes.

In Excel 2003 running in XP, go to the Tools menu > Macro > Security (Figure A-3).

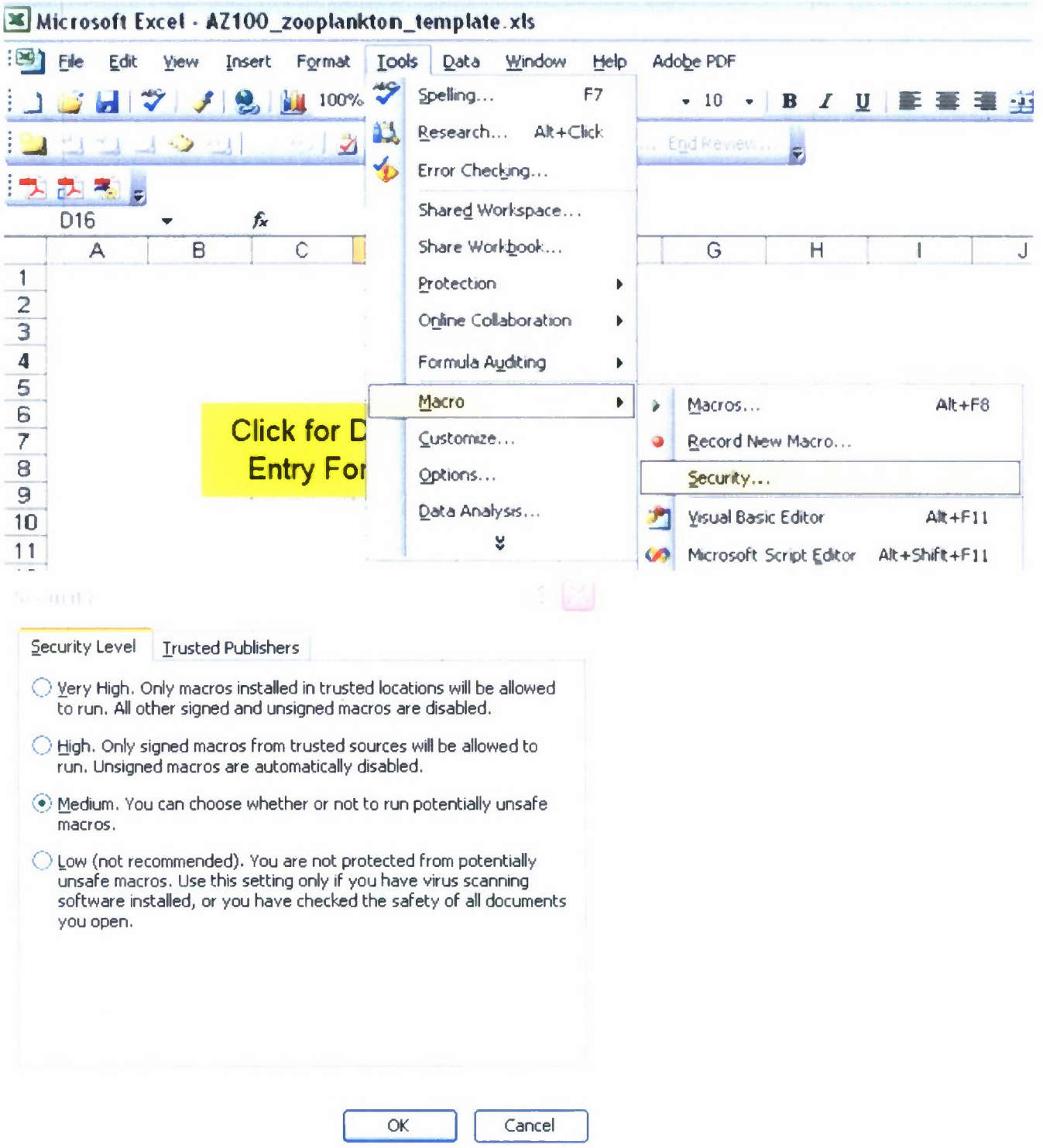

Figure A-3. Excel data sheet security menu (A-3A) and security selection level (A-3B).

Choose Medium security (Figure A-3B), click OK, close Excel, and reopen it.

3. On the Data Entry worksheet, click on the yellow Data Entry button (Figure A-4).

Figure A-4. Data entry form button.

Entries or checks are required for all data fields (otherwise, the file cannot be saved). See Figure A-5.

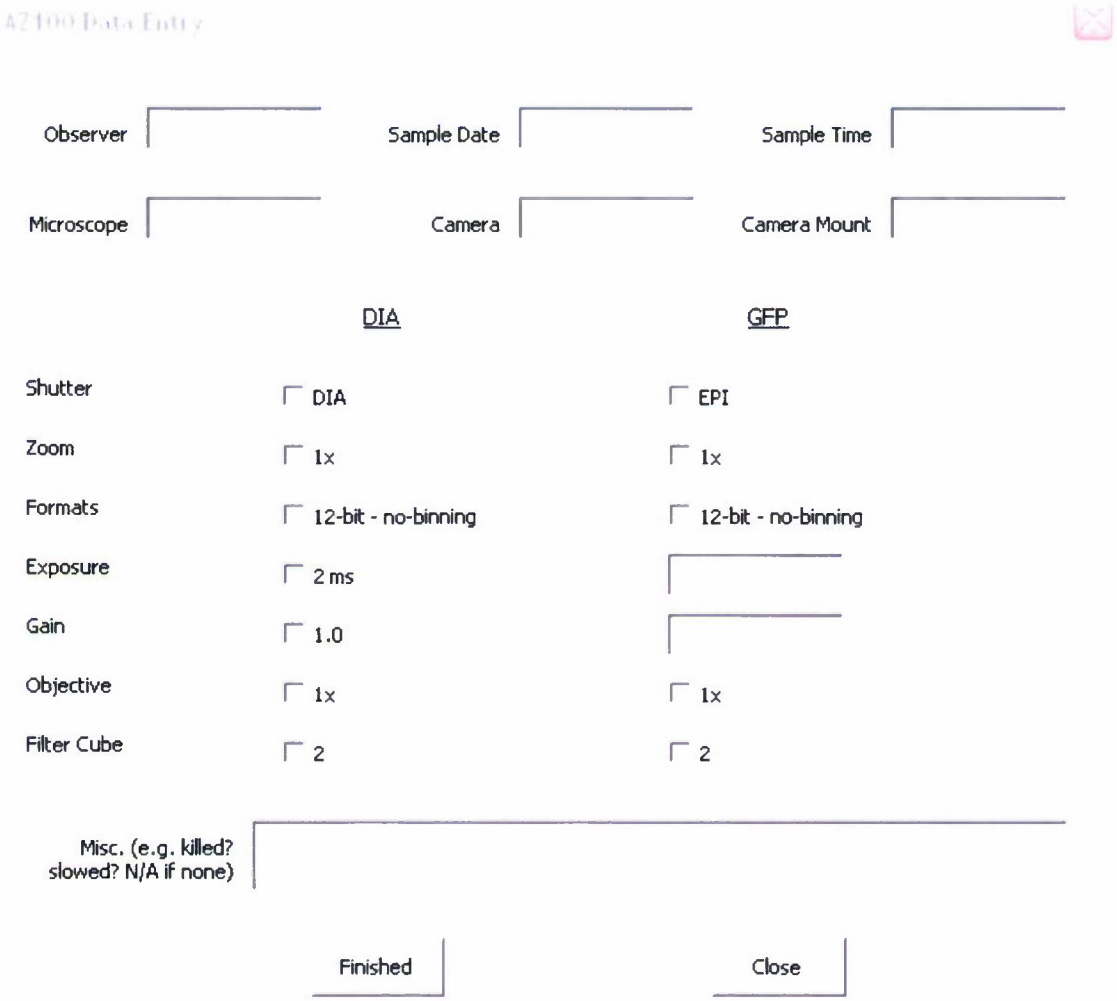

Figure A-5. Required data fields in Excel data sheet.

4. After each .nd2 file is recorded, view the images and write observations in the appropriate 'Comments' worksheet in the Excel file (Figure A-6).

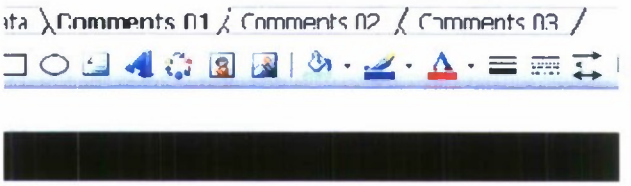

Figure A-6. Excel data sheet comment pages for each .nd2 file.

A.3 ZOOPLANKTON DATA COLLECTION PROTOCOL

A.3.1 Initial Set-up

1) Before opening Elements program, turn on the camera (RGB filter set to MONO) and both lamps.
2) Place your sample on the stage and focus the microscope using the oculars. Once the sample is in focus, pull out the knob to change the optical pathway from the oculars to the camera.
3) In Elements, open up a live preview. You can do this by going to the Acquire menu (Live – Fast), by clicking the "Play" Icon on the toolbar (see Figure A-7), or by pressing the '+' key on the keyboard.

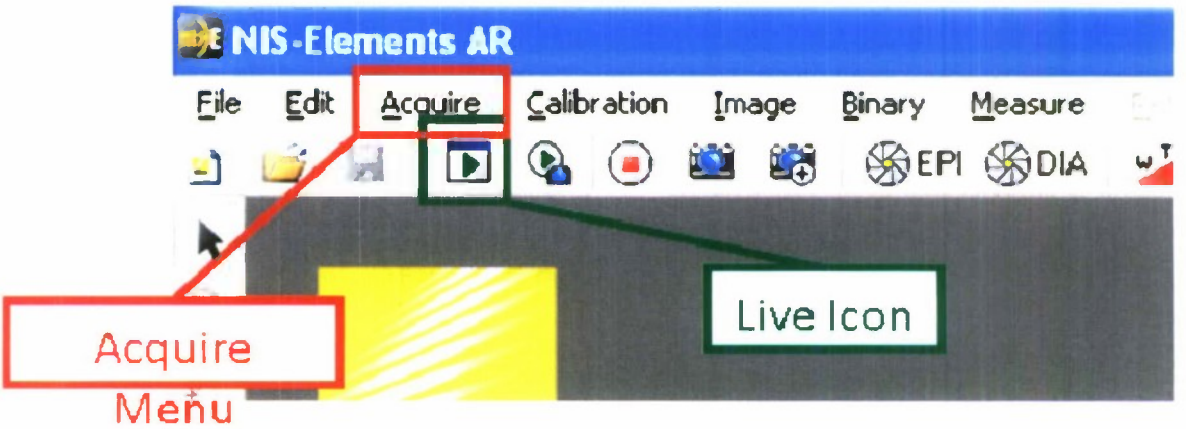

Figure A-7. Acquire menu and Live icon.

A.3.2 Camera Set-up

4) Click on the DIA optical configuration in the toolbar (red box, Figure A-8). Make sure it is not the shutter button (purple box, Figure A-8).

   The optical configuration buttons are preset with settings for the camera exposure, image gain, objective and filter cube, etc.

   These are the settings that will be used when you take a time series of images.

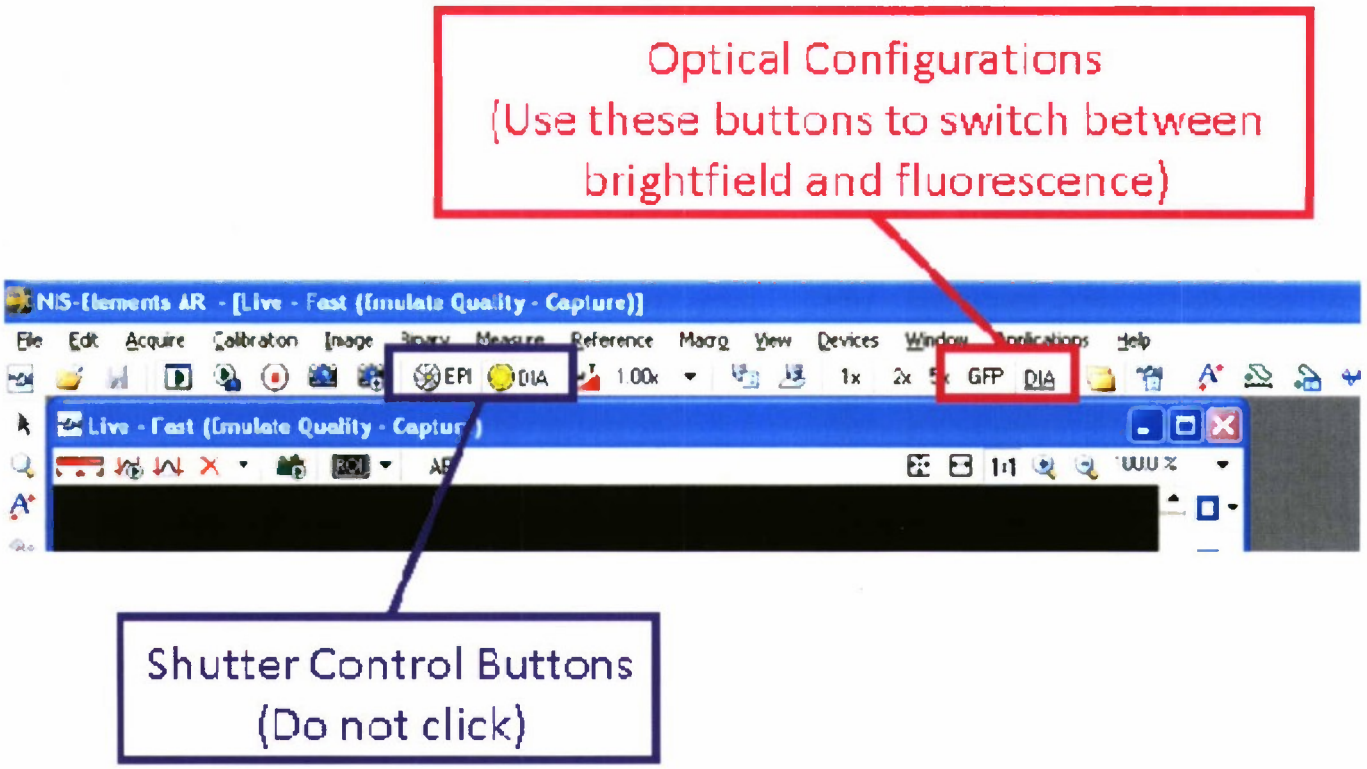

Figure A-8. Optical configuration and shutter control buttons.

A.3.3 Camera Focus and Settings

5) Now use the focus ring on the coupler to focus the camera based on the Live Preview window. You may need to zoom in on the Preview to make sure it is as crisp as possible. (Figure A-9).

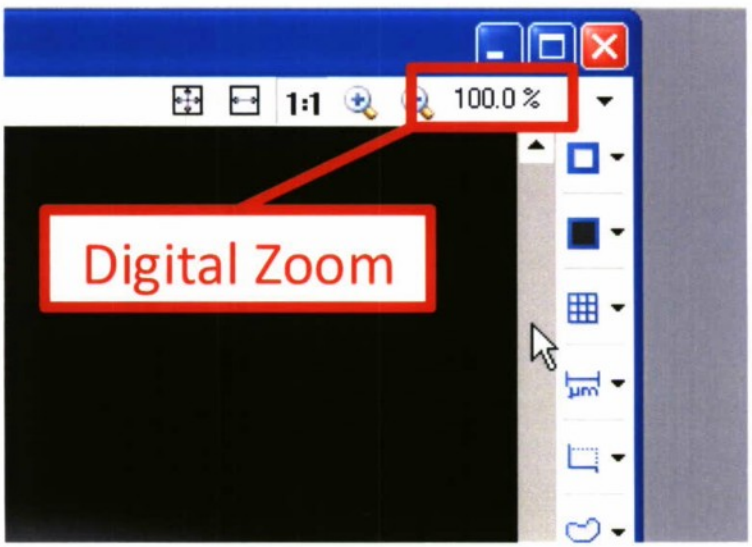

Figure A-9. Digital zoom button.

6) Check the physical microscope settings:
   - The GFP Band Pass filter cube is in place (2)
   - The 1x objective is in place.
   - Zoom is at 1x.
   - At the bottom right of the base, the ND16 and ND8 filter knobs are pulled out (keep the ND2 knob pushed in; n.b. ND = neutral density).

A.3.4 Verification of Elements Settings - DIA

7) Check all your Elements settings. With the DIA optical configuration button (orange box, Figure A-10) selected, there are several things to look for (Figure A-11):
   i. The DIA shutter is selected.
   ii. The zoom is 1x.
   iii. The formats for live and for capture are both set at 12-bit - no binning.
   iv. The exposure time is 2 ms.
   v. The Hardware Gain is 1.0.
   vi. On the Manual Microscope Pad menu, the 1x nosepiece is selected.
   vii. The Dia filter turret is selected.

(Note that some of these settings may change over time and depending on the sample.)

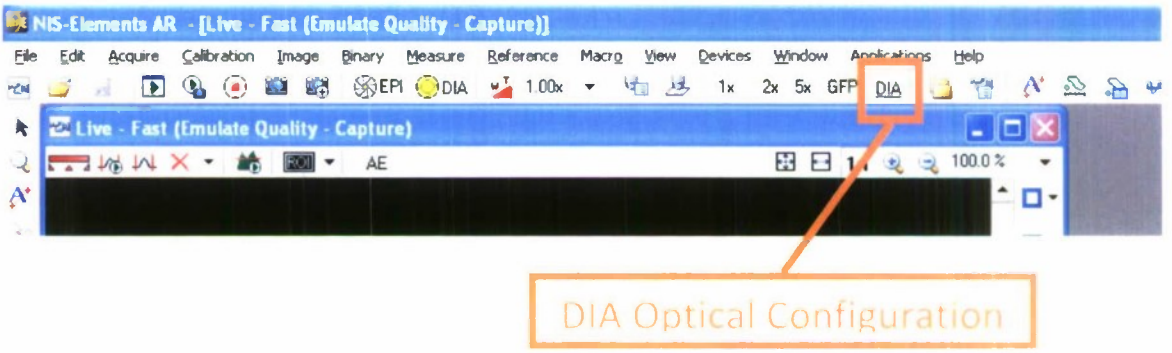

Figure A-10. DIA optical configuration button.

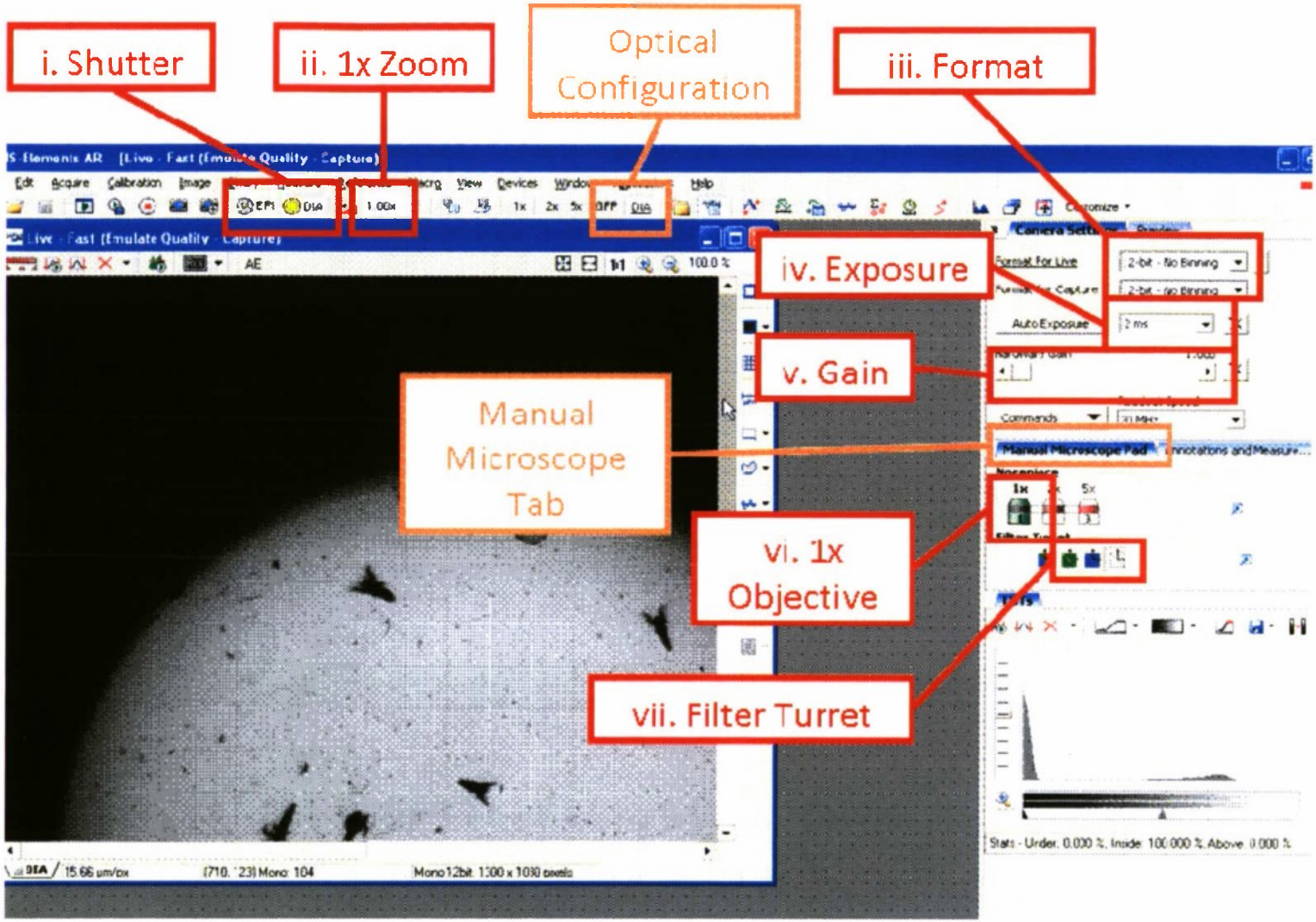

Figure A-11. DIA setting summary.

A.3.5 Verification of Elements Settings - GFP

8) Now click on the GFP Optical Configuration button (green box, Figure A-12) and double check the GFP settings:

- The EPI shutter button is selected.
- The zoom is 1x.
- The formats for live and for capture are both set at 12-bit - no binning.
- The exposure time is 300 ms.
- The Hardware Gain is 5.0.
- On the Manual Microscope menu, the 1x nosepiece is selected.
- The ETGFP filter turret is selected.

(Note that some of these settings may change over time and depending on the sample.)

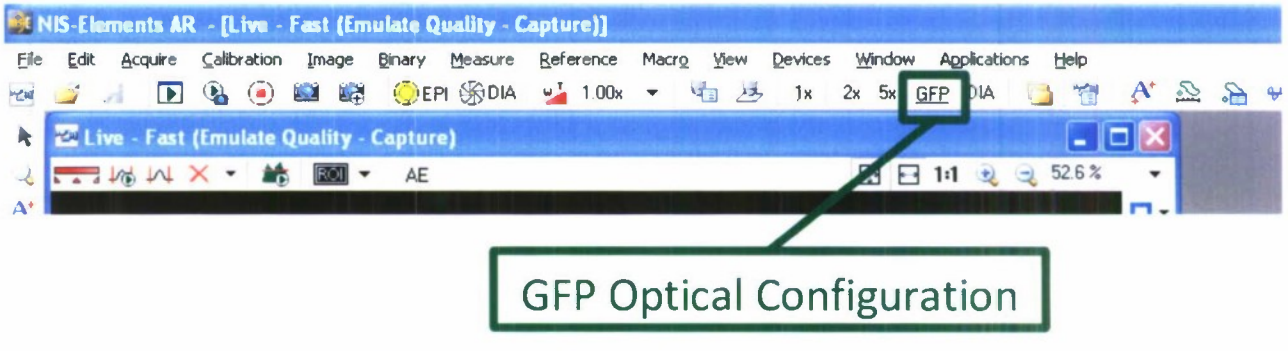

Figure A-12. GFP optical configuration.

If you make changes to the exposure or gain while an Optical Configuration is selected, the new values will be propagated as part of the preset values. That is why you need to make sure the settings are correct before beginning data collection.

A.3.6 Manual Changes

You can reset the presets by manually changing the above settings, going to the Calibration menu at the top of the screen, and choosing Optical Configurations. Make sure the correct optical configuration is selected in the menu to the left and then click on "Assign Current Camera Settings" or "Assign Current Microscope Settings" (Figure A-13).

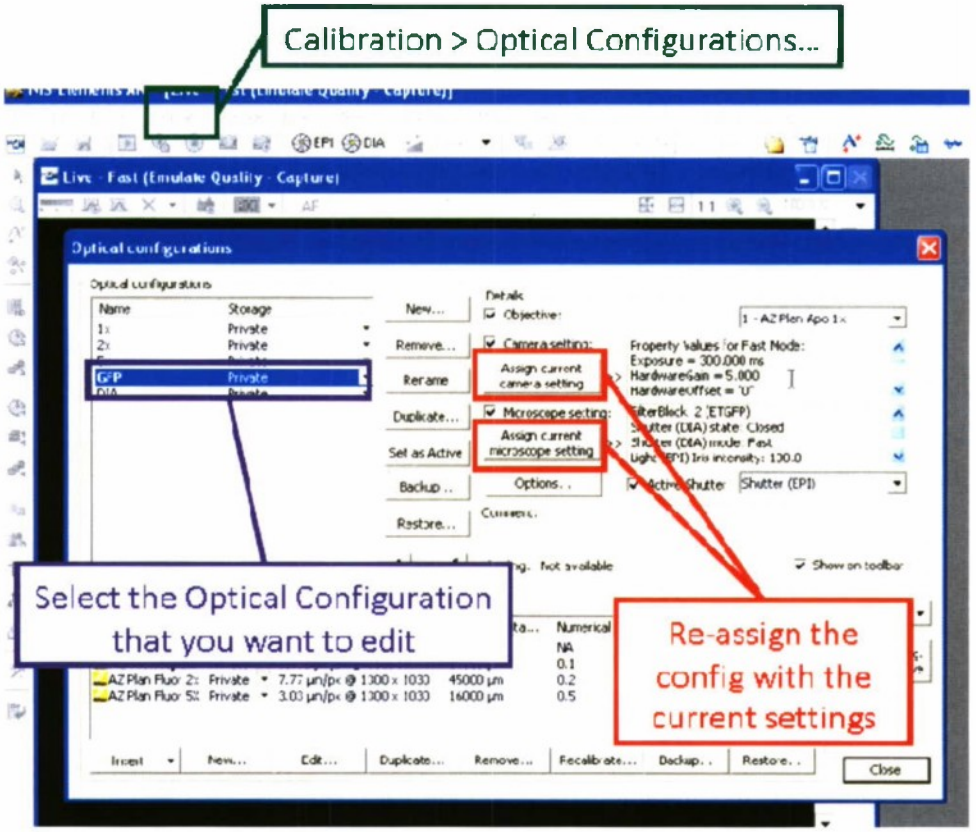

Figure A-13. Assign current settings.

A.3.7 Final Step - Designating Acquisition File

9) At this point, you have checked all your settings and the camera is in focus. Go to the Applications menu and choose Define/Run Experiment (Figure A-14).

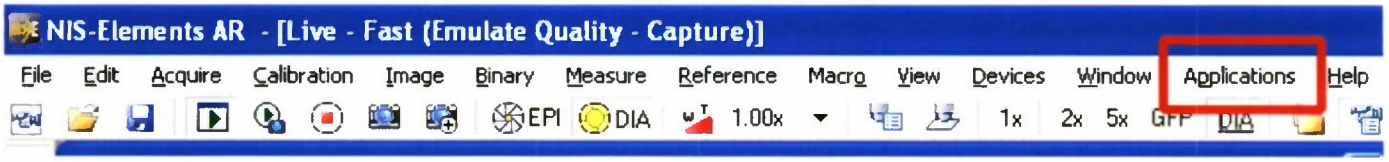

Figure A-14. Applications button.

10) Click on Browse to choose which folder to save the images in. Enter your file name_001 (Elements will automatically increase the number at the end of each run). Set the Interval to 1 sec and the Duration to 30 sec. The Loops will automatically change to 31. See Figure A-15.

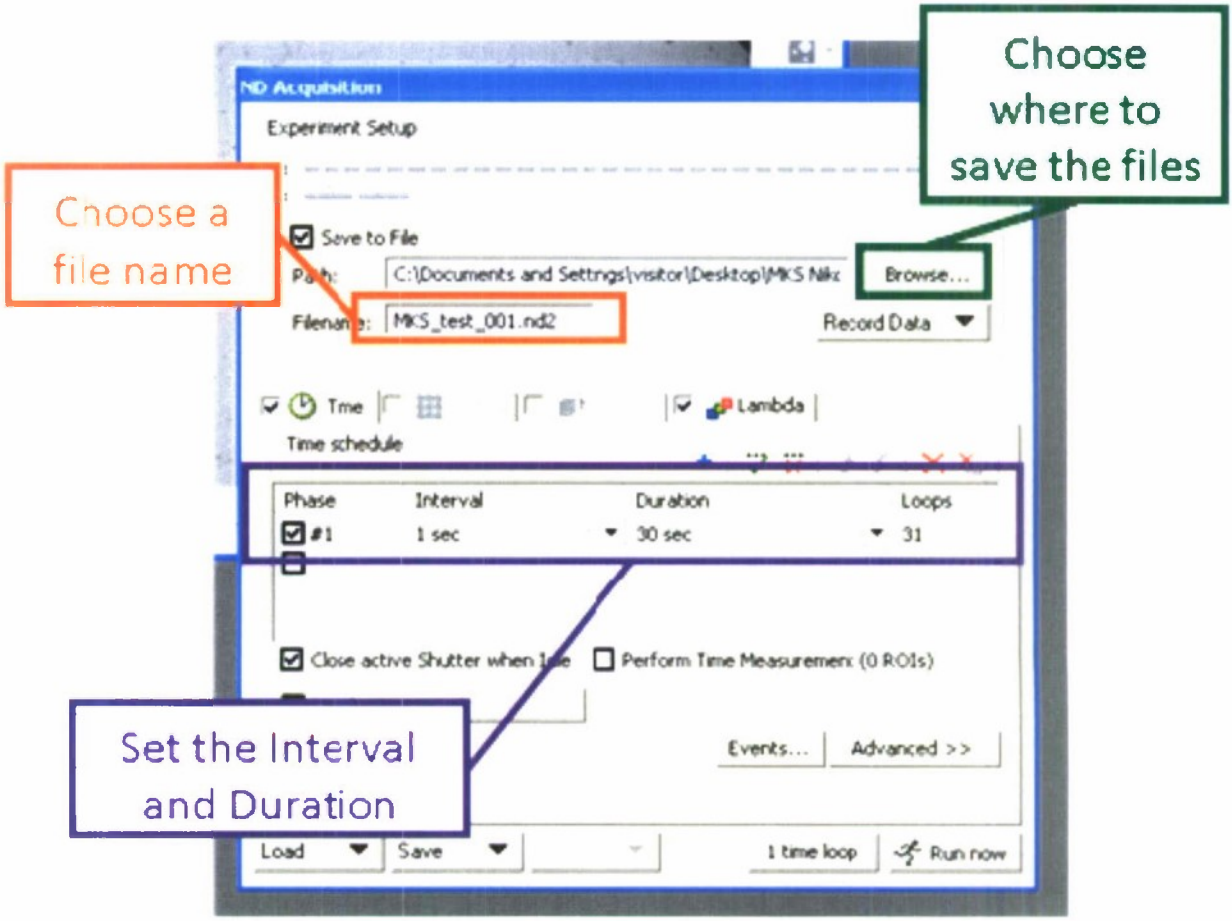

Figure A-15. File designation set-up.

A.3.8 Acquire Data

11) Click on the Lambda tab (Figure A-16). Make sure the first Lambda is GFP and the second Lambda is DIA.

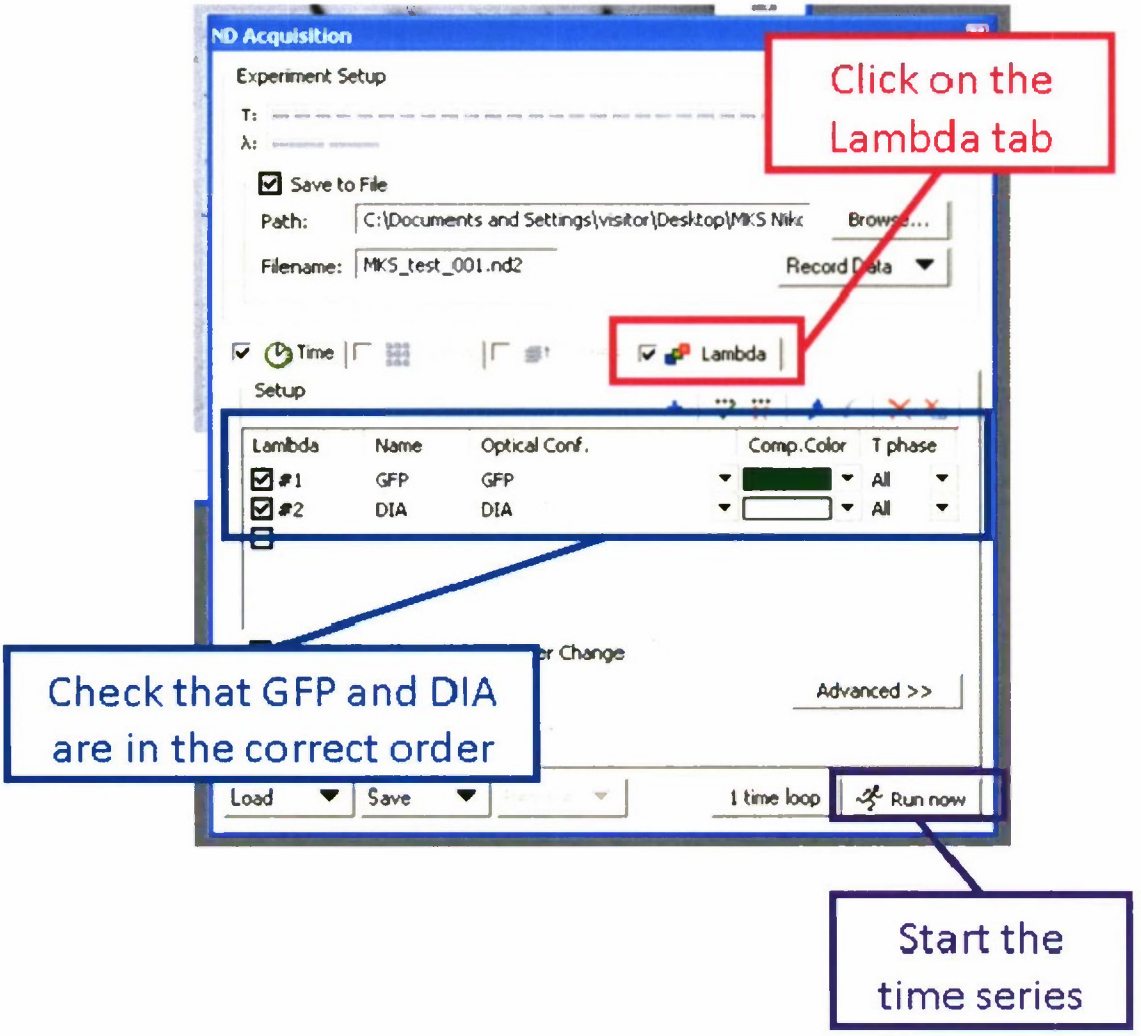

Figure A-16. Setting light source sequence.

12) Everything is set up, so click "Run now" to start the time series (Figure A-16).